Volume 282, number 1, 135–138 FEBS 09638 April 1991

ADONIS 001457939100346T

NADH:ubiquinone oxidoreductase from bovine heart mitochondria

Complementary DNA sequence of the import precursor of the 10 kDa subunit of the flavoprotein fragment

J. Mark Skehel, Stephanie J. Pilkington, Michael J. Runswick, Ian M. Fearnley and John E. Walker

Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

Received 25 February 1991

The amino acid sequence of the 10 kDa subunit of the flavoprotein (FP) fragment of complex I from bovine heart mitochondria has been determined by protein sequence analysis, thereby completing the sequence of the FP fragment. The calculated molecular weight of the 10 kDa subunit agrees exactly with the value of 8438 determined by electrospray mass spectrometry, and further confirmation of the sequence has been obtained by sequencing cDNAs amplified from total bovine heart cDNA by the polymerase chain reaction, using mixed oligonucleotides based upon the protein sequence as primers and hybridization probes. The sequence of the 10 kDa subunit is not related to that of any known protein. Being devoid of cysteine residues, it has none of the characteristic features of known iron-sulfur proteins and it is improbable that it is involved in liganding Fe-S centers in the FP fragment.

Complex I (bovine heart mitochondria); Flavoprotein fragment; 10 kDa Subunit

1. INTRODUCTION

NADH:ubiquinone oxidoreductase (complex I) from bovine heart mitochondria is a membrane-bound assembly of more than 30 different polypeptides that has been split into subcomplexes with chaotropes [1–3]. The flavoprotein (FP) fragment, a water-soluble complex of the 51, 24 and 10 kDa subunits in unit stoichiometry, can transfer electrons from NADH to ferricyanide, but not to coenzyme Q [3–5]. It contains FMN, the primary electron acceptor from NADH, and a 4Fe-4S and a 2Fe-2S iron-sulfur center, designated N-3 and N-1b, respectively, have been assigned to it [3,6]. The binding sites for NADH and FMN, and the 4Fe-4S center are all believed to be associated with the 51 kDa subunit, and the binuclear center is thought to be associated with either the 24 or the 10 kDa subunit, or possibly with both [3]. The sequence of the 51 kDa subunit [7] contains the motif CysXXCysXXCys, which provided 3 of the 4 cysteine ligands of 4Fe-4S centers in bacterial ferredoxins, for example, and so provides further evidence for the presence of a 4Fe-4S center. The sequence of the 24 kDa subunit is also known [8]. Its 5 cysteine residues are conserved in mammals, but the sequence CysXXXXCysXXXCys, which is associated with bacterial 2Fe-2S centers, is not present, and only 2 of the 5 conserved cysteines are found in a homologous protein from a bacterial NAD^+ reducing hydrogenase [7,9]. In order to investigate the possibility that the 10 kDa subunit of the bovine enzyme could provide cysteine ligands for a binuclear center, as described below, we have determined its primary structure both by protein sequencing and by sequencing its cDNA. These experiments show that the mature 10 kDa subunit contains no cysteine residues, and there is no evidence in the sequence for its participation in liganding a 2Fe-2S center.

Correspondence address: J.E. Walker, Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK. Fax: (44) (223) 412178

2. MATERIALS AND METHODS

2.1. *Isolation and protein sequencing of the 10 kDa subunit*

Complex I was purified from bovine heart mitochondria [10]. The FP fragment was prepared as described before [2,11], and its subunits were separated by gel electrophoresis, transferred to a poly(vinylidene difluoride) membrane and sequenced at their N-terminals. The 10 kDa subunit was also isolated from a water soluble fraction of complex I obtained by dissociation of the enzyme in 6 M guanidine hydrochloride, followed by dialysis against 0.4% ammonium bicarbonate solution. A precipitate was removed by centrifugation and the supernatant (the water soluble fraction) was fractionated by HPLC on an Aquapore C_8 column (300 Å pore size, 7 μ particles; 100 mm × 2.1 mm i.d., Applied Biosystems, Warrington, UK) in 0.1% trifluoroacetic acid with an acetonitrile gradient. The 10 kDa subunit eluted at 34% acetonitrile. Digests of the protein with trypsin, chymotrypsin and endoproteinase Asp-N were fractionated by HPLC as described above. Peptides and purified protein were sequenced in an Applied Biosystems gas phase sequencer [12].

2.2. *Electrospray mass spectrometry*

Spectra were measured with a VG BIO-Q triple quadrupole instrument with electrospray ionization (VG Biotech, Altrincham, Cheshire, UK). Samples of the 10 kDa protein (ca. 100 pmol dissolved

in 10 μl of a solution of 1% acetic acid in 50% aqueous methanol) were delivered from an Applied Biosystems 120 liquid chromatograph at 4 μl/min and sprayed in the same solvent. The mass spectrum was obtained from scans of mass to charge ratios between 600 and 1600 over 10 s intervals. The instrument was calibrated with horse heart myoglobin.

2.3. Isolation and sequencing of cDNA clones for the 10 kDa subunit

cDNAs for the 10 kDa subunit were amplified from total bovine heart cDNA by the polymerase chain reaction (PCR) using mixtures of synthetic oligonucleotides based upon the known protein sequence as primers and hybridization probes. Details of similar experiments conducted on other subunits of complex I and of DNA sequence analysis have been described elsewhere [7,13,14]. See also the legend to Fig. 1 for additional details. The DNA sequence was determined completely in both senses of the DNA.

3. RESULTS AND DISCUSSION

3.1. *Protein sequence of the 10 kDa subunit of complex I*

The N-terminal sequence of the 10 kDa protein present in the FP fraction was determined up to the 25th residue and the remainder of its primary structure was deduced from the sequences of overlapping peptides isolated from enzymic digests of the protein (see Fig. 1). The mature 10 kDa subunit of the FP fraction of bovine complex I is 75 amino acids long, and its composition calculated from the sequence (see Table I) is in reasonable general agreement with that determined previously on the isolated protein [2], although their are some discrepancies, particularly in the glycine content. Also, the question of whether the protein contained any cysteine residues was unresolved by the protein sequencing experiments, because no attempt had been made to identify such residues. However, values of molecular mass of the protein measured by electrospray mass spectrometry agree exactly with the value of 8438 calculated from the sequence (see Table I). Therefore, this confirmed that the sequence was likely to be correct and that cysteine residues were absent from the 10 kDa subunit. Further confirmation of the mature protein sequence and the absence of cysteine residues came from the cDNA sequence (see below).

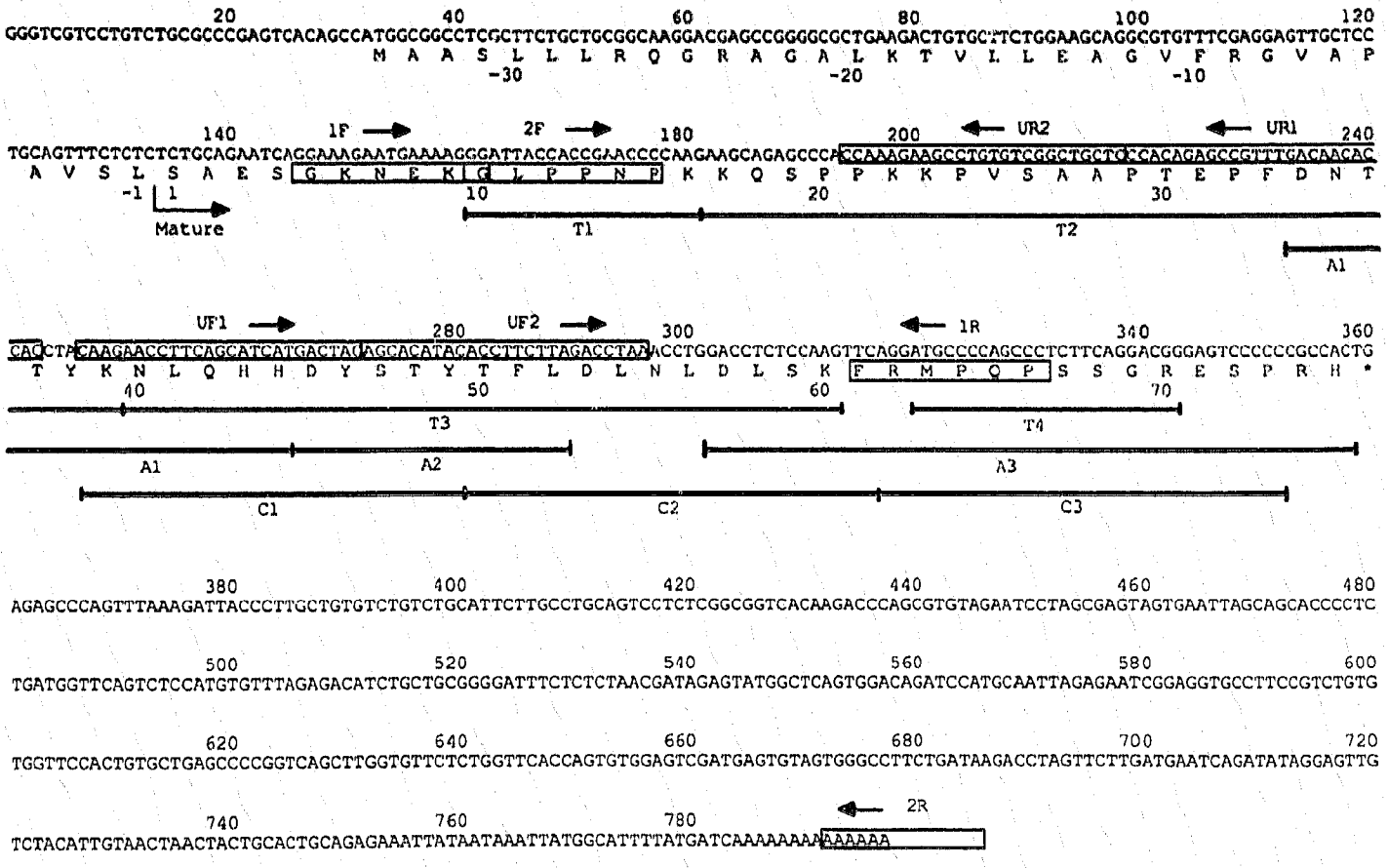

Fig. 1. Analysis by protein and DNA sequencing of the 10 kDa subunit of complex I from bovine heart mitochondria. The sequence of residues 1–25 of the mature protein was determined by N-terminal analysis. The remainder was deduced from overlapping peptides isolated from enzymic digests with trypsin (T1–T4), endoproteinase Asp-N (A1–A3), and chymotrypsin (C1–C3). The DNA sequence was determined by means of a strategy based on the polymerase chain reaction, and the positions of protein sequences used in the design of mixed oligonucleotide primers and the positions of unique oligonucleotide primers are boxed. The arrows indicate the senses of the various primers. For further details see the text. The DNA sequence also encodes an N-terminal extension (amino acids −1 to −34) not present in the mature protein.

Table I

Amino acid composition and molecular mass of the 10 kDa subunit of the flavoprotein fragment of bovine complex I

Amino acid	Number of residues	
	By analysis[1]	From sequence
aspartic acid	10.1[2]	5
asparagine	–	4
threonine	5.6	5
serine	7.7	9
glutamic acid	8.3[3]	4
glutamine	–	3
proline	12.1	11
glycine	7.4	3
alanine	4.0	3
valine	1.9	1
methionine	0.9	1
isoleucine	0.7	0
leucine	7.1	6
tyrosine	3.0	3
phenylalanine	3.0	3
histidine	3.2	3
lysine	8.3	8
arginine	4.0	3
cysteine	1.0	0
tryptophan	0	0
Total		75
Molecular mass		8438[4]

[1]Recalculated from [2]. [2]Sum of aspartic acid and asparagine residues. [3]Sum of glutamic acid and glutamine residues. [4]Calculated from the sequence. The values obtained in two electrospray mass spectrometry experiments were 8438.55 and 8438.34 using 6 z [z = 7–12]. The standard deviations of these measurements were 0.26 and 0.38, respectively.

3.2. *cDNA sequence encoding a mitochondrial import precursor of the 10 kDa subunit*

The amplification of cDNAs encoding the 10 kDa subunit of complex I was carried out in 3 stages. Firstly, a cDNA encoding the central part of the protein (amino acids 5–66) was amplified by the polymerase chain reaction using two overlapping mixed oligonucleotides based on amino acids 5–10 and 10–15 as forward primers (primers 1F and 2F in Fig. 1), and a third mixed oligonucleotide based on amino acids 61–66 as reverse primer. The desired product was identified by hybridization with a mixed 17-mer oligonucleotide based on amino acids 46–51. Then parts of the sequence of this product were used to make unique primers (UF1 and UF2; UR1 and UR2) and unique probes (nucleotides 297–313, and the complement of nucleotides 177–194), and these were used in further PCR experiments to obtain cDNAs extending to the 3′ and 5′ extremities, respectively. In both cases, oligo dT was used as one of the primers; in the former case (see primer 2R in Fig. 1) it hybridized with the 3′ poly A tail derived from the mRNA, and in the latter case with poly A that had been added with terminal transferase to the 5′ end of the bovine heart cDNA population. These 3 partial cDNAs together constitute a cDNA encoding a precursor of the 10 kDa subunit. Assuming that nucleotides 32–34 are the initiator ATG codon, the mitochondrial import precursor is 34 amino acids long and in common with other mitochondrial import sequences it has a net positive charge. Most importantly, the cDNA sequence confirms that the sequence of the mature 10 kDa subunit of the flavoprotein fraction of complex I contains no cysteine residues. The coding sequence is followed at its 3′ end by a non-coding sequence of 423 nucleotides. This contains the sequence AATAAA, a typical polyadenylation signal [15], with 18 intervening nucleotides before the 3′ poly A tail.

3.3. *The iron-sulfur centers of the FP fragment of complex I*

The FP fragment of bovine complex I has been reported to contain 6 iron atoms [2,3,11], and EPR studies of the isolated fragment suggest the presence of a tetranuclear and a binuclear center [3], probably the centers known as N-3 and N-1b, respectively. The evidence for the location of the tetranuclear center in the 51 kDa subunit and of the binuclear center in either the 24 or 10 kDa subunits or in both has been summarized above, and the sequence of the 51 kDa subunit agrees with the presence of a tetranuclear center [7]. The sequences of the bovine 24 [8] and 10 kDa subunits show that 5 cysteine residues are in the former and that the latter has none. Therefore, if the binuclear center is liganded exclusively by cysteine residues, the 10 kDa subunit does not contribute and the cysteine ligands must be in the 24 kDa subunit. Although the sequence CysXXXXCysXXXCys is not present in the 24 kDa subunit, its 5 cysteines are conserved in mammals suggesting some functional importance for them, including possibly formation of a binuclear center.

Additional information about the possible roles of these cysteines comes from the sequence of a bacterial NAD^+ reducing hydrogenase. The NAD^+ reducing part of this enzyme is made of two subunits (α and γ) and has a number of similarities to mitochondrial complex I [7]. Most significantly for the present discussion, the α subunit is a fusion of homologues of the 24 and 51 kDa subunits of complex I (in the same order N- to C-terminal). Also, the γ subunit is homologous throughout its sequence to the N-terminal region of the 75 kDa subunit of complex I. The $\alpha\gamma$-dimer has been proposed to contain 2 tetranuclear centers and a binuclear center [9,16], with the former both in the γ-subunit and the binuclear center in the α-subunit. However, the region of the α-subunit which is related to the 24 kDa subunit contains in conserved positions only 2 of the 5 cysteines found in the mammalian 24 kDa subunits of complex I. Therefore, the simplest interpretation is that the bacterial enzyme and the FP portion of complex I have different arrangements of the binuclear iron-sulfur centers, despite the homologies between the 24 and α-subunits. Other explanations are also possible. For example, that the binuclear centers in

the complex I 24 kDa subunit and the hydrogenase α-subunit are liganded by the 2 conserved cysteines and 2 non-cysteine ligands. The C-terminal region of the hydrogenase α-subunit, and the related 51 kDa subunit of complex both contain the motif CysXXCysXXCys, and on the basis of the studies in the FP fraction of complex I, it seems likely that this binds a tetranuclear center, although no such center was proposed on the basis of the sequence to be present in this region of the hydrogenase α-subunit [9]. Further investigation of these centers in both enzymes are needed to clarify these matters.

Acknowledgements: We thank Mr. T.V. Smith and Mrs. J. Fogg for synthesizing oligonuleotides. S.J.P. was supported by an MRC research studentship and an MRC research training fellowship.

REFERENCES

[1] Hatefi, Y. and Stempel, K.E. (1969) J. Biol. Chem. 244, 2350-2357.
[2] Galante, Y.M. and Hatefi, Y. (1979) Arch. Biochem. Biophys. 192, 559-568.
[3] Ragan, C.I. (1987) Current Topics Bioenerget. 15, 1-36.
[4] Dooijewaard, G. and Slater, E.C. (1976) Biochim. Biophys. Acta 440, 1-15.
[5] Dooijewaard, G. and Slater, E.C. (1976) Biochim. Biophys. Acta 440, 16-35.
[6] Ohnishi, T., Ragan, C.I. and Hatefi, Y. (1985) 260, 2782-2788.
[7] Pilkington, S.J., Skehel, J.M., Gennis, R.B. and Walker, J.E. (1991) Biochemistry 30, 2166-2175.
[8] Pilkington, S.J. and Walker, J.E. (1989) Biochemistry 28, 3257-3264.
[9] Tran-Betcke, A., Warnecke, U., Böcker, C., Zabarosch, C. and Friedrich, B. (1990) J. Bacteriol. 172, 2920-2929.
[10] Ragan, C.I., Wilson, M.T., Darley-Usmar, V.M. and Lowe, P.N. (1987) in: Mitochondria: A Practical Approach (Darley-Usmar, V.M., Rickwood, D. and Wilson, M.T. eds.) pp. 79-112, IRL Press, Oxford.
[11] Ragan, C.I., Galante, Y.M., Hatefi, Y. and Ohnishi, T. (1982) Biochemistry 21, 590-594.
[12] Fearnley, I.M., Runswick, M.J. and Walker, J.E. (1989) EMBO J. 8, 665-672.
[13] Dupuis, A., Skehel, J.M. and Walker, J.E. (1991) Biochem. J., in press.
[14] Dupuis, A., Skehel, J.M. and Walker, J.E. (1991) Biochemistry 30, 2954-2960.
[15] Proudfoot, N.J. and Brownlee, G.G. (1976) Nature 263, 211-214.
[16] Schneider, K., Cammack, R. and Schlegel, H.G. (1984) Eur. J. Biochem. 142, 75-84.